INT J TUBERC LUNG DIS 3(9):830–837

DJ

Direct detection and identification of *Mycobacterium tuberculosis* in smear-positive sputum samples by fluorescence in situ hybridization (FISH) using peptide nucleic acid (PNA) probes

H. Stender,* T. A. Mollerup,* K. Lund,* K. H. Petersen,* P. Hongmanee,† S. E. Godtfredsen*

* DAKO A/S, Glostrup, Denmark, † Department of Pathology, Ramathibodi Hospital, Bangkok, Thailand

SUMMARY

SETTING: Peptide nucleic acid (PNA) probes designed for specific detection of mycobacteria of the *Mycobacterium tuberculosis* complex (MTC) and other non-tuberculous mycobacterium species (NTM) are shown to be able to penetrate the mycobacterial cell wall and subsequently hybridize in situ to complementary rRNA.

OBJECTIVE: To demonstrate the use of fluorescein-labelled PNA probes for detection and identification of *M. tuberculosis* in smear-positive sputum samples.

DESIGN: The sensitivity and specificity of the PNA probes were investigated by fluorescence in situ hybridization (FISH) using cultures of mycobacterium strains representing species of the MTC and NTM, respectively.

RESULTS: *M. tuberculosis* strains were detected by FISH using specific fluorescein-labelled PNA probes directly in smear-positive sputum samples without changing the morphology of the cells.

CONCLUSION: PNA probes allow for rapid diagnosis of tuberculosis in smear-positive cases.

KEY WORDS: PNA: TB; FISH; diagnosis; *Mycobacterium tuberculosis*

TODAY TUBERCULOSIS is still the largest cause of death caused by single infections.[1,2] Individuals with pulmonary tuberculosis are highly infectious and require immediate isolation and initiation of antituberculosis therapy.[3] Early laboratory diagnosis of tuberculosis relies on an initial microscopic examination of sputum smears stained for acid-fast bacilli (AFB), the presence of which is the first indication of possible *Mycobacterium tuberculosis* infection. The final diagnosis, however, must await species identification, which in many cases involves several weeks of culturing.[4]

In recent years, molecular methods based on target amplification have become available for identification of *M. tuberculosis* in smear-positive sputum samples. Molecular methods based on in situ hybridization of labelled probes to microbiological targets are considerably different, as they do not require amplification of the target prior to detection. Thus detection and identification of mycobacterium species are performed directly on the sputum samples.

Peptide nucleic acids (PNAs) are pseudo-peptides with DNA-binding capability. These compounds were first reported in the early 1990s in connection with a series of attempts to design nucleotide analogues capable of hybridizing, in a sequence-specific fashion, to DNA and RNA.[5–7]

Hybridization of PNA to DNA and to RNA has been shown to obey the Watson-Crick base pairing rules, and PNA has been found to hybridize to a DNA or RNA target with higher affinity and specificity than their nucleic acid (NA) counterparts.[8] These properties are ascribed to the uncharged structure of the PNA backbone, and to the high conformational flexibility of the PNA molecules.[7]

The relative hydrophobic character of PNA as compared to DNA and RNA might be particularly useful in diagnostic applications where access of detection probes to their molecular target will depend upon efficient diffusion of the probe through a hydrophobic environment, such as a cell wall, under sufficiently mild conditions for the morphology of the cell to be preserved. Maintenance of cell morphology is particularly important for applications where simple microscopic examination of a specimen is desirable. One such example of a potentially important, so far unexplored, diagnostic application is the use of PNA for direct, specific detection of *M. tuberculosis* in sputum samples where maintenance of bacterial morphology is a crucial factor for microscopic examination. Applied probes must therefore be able to diffuse through the hydrophobic cell wall of mycobacteria under conditions which do not lead to disruption of the bacterial morphology.[9]

Correspondence to: Henrik Stender, Microbiology Department, DAKO A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark. Tel: (+45) 44 85 95 00. Fax: (+45) 44 92 00 56.

Article submitted 19 January 1998. Final version accepted 24 March 1999.

DNA probes have been tested for in situ detection of mycobacterium species, but this application has not been developed into a practical diagnostic test.[10,11]

In the present paper we present the use of PNA probes for specific detection of mycobacteria of the *M. tuberculosis* complex (MTC) and other non-tuberculous mycobacterium (NTM) species by fluorescence in situ hybridization. The method is applied for detection of *M. tuberculosis* directly in sputum smears. The results obtained suggest that this new molecular method for laboratory diagnosis of tuberculosis is adaptable with current microscope techniques routinely used in the majority of clinical microbiology laboratories, hereby allowing them to make a final confirmation of *M. tuberculosis* on smear-positive cases.

MATERIALS AND METHODS

Clinical specimens

Two smear-positive sputum specimens from patients with clinical signs of tuberculosis were collected at the Ramathibodi Hospital, one of five medical centres in Bangkok, Thailand, serving a metropolitan population with a high prevalence of tuberculosis.[12]

Preparation of smears

Sputum specimens were smeared onto microscope slides and flame-fixed according to standard procedures for acid-fast staining.[4] Further inactivation of mycobacteria was carried out by incubating the slides at 80°C for 2 hours in a laboratory incubator. Thereafter, the slides were slowly cooled overnight to room temperature by switching off the incubator.

Mycobacterium species

M. tuberculosis (ATCC no. 25177), *M. bovis* BCG (ATCC no. 35734), *M. avium* (ATCC no. 25292), *M. intracellulare* (ATCC no. 13950), and *M. kansasii* (ATCC no. 12479) grown on Dubos broth (Statens Serum Institut, Copenhagen, Denmark) were used as controls. The cultures were sedimented by centrifugation and subsequently resuspended in phosphate buffered saline (PBS), smeared onto microscope slides, and flame-fixed.

PNA synthesis and labelling

PNA probes were synthesized by use of an Expedite 8909 Nucleic Acid Synthesis System purchased from PerSeptive Biosystems (Framingham, MA, USA). The PNA oligomers were terminated with either two β-alanine or one lysine and, before cleavage from the resin, labelled with 5(6)-carboxyfluorescein at the β-amino group of alanine or ε-amino group of lysine, respectively. Probes were purified by reverse phase high performance liquid chromatography (HPLC) at 50°C and characterized using a G 2025 A MALDI-TOF MS instrument (Hewlett Packard, San Fernando, CA, USA). The molecular weights determined were within 0.1% of the weights calculated.

In situ hybridization

Smears were immersed in 80% (v/v) ethanol for 15 minutes and subsequently air-dried. Air-dried smears were covered with approximately 20 μL of hybridization solution containing 10% (w/v) dextran sulphate (Sigma Chemical Co., St. Louis, MO, USA), 10 mM NaCl (Merck, Darmstad, Germany), 30% (v/v) formamide (Life Technologies, Gaithersburg, MD, USA), 0.1% (w/v) sodium pyrophosphate (Merck), 0.2% (w/v) polyvinylpyrrolidone (Sigma), 0.2% (w/v) ficoll (Sigma), 5 mM Na_2EDTA (Merck), 0.1% (v/v) Triton X-100 (Serva, Heidelberg, Germany), 50 mM Tris/HCl pH 7.5, 1 μM of a non-labelled, non-target PNA probe, and 25–100 nM fluorescein-labelled PNA probe (see Figure legend for concentration). Coverslips were put on the samples in order to ensure even coverage with the hybridization solution and were subsequently placed in a moist chamber and incubated for 1.5 hours at 55°C. Handling of the smears and solutions was carried out using gloved hands to avoid ribonuclease contamination. Whenever possible, equipment was heat-treated, and solutions were exposed to 1 μL/mL diethyl-pyrocarbonate (Sigma) in order to inactivate nucleases.[13]

Following hybridization, the coverslips were removed and the slides were submerged into prewarmed 5 mM Tris/HCl, 15 mM NaCl, 0.1% (v/v) Triton X-100 (pH 10) in a waterbath at 55°C and washed for 30 min. The slides were then cooled to room temperature by brief immersion in H_2O.

The sputum smears were finally mounted with one drop of IMAGEN™ Mounting Fluid (DAKO, Glostrup, Denmark) and covered with coverslips.

Microscope examinations were conducted using a fluorescence microscope (Leica, Wetzlar, Germany) equipped with a 100×/1.30 oil objective, an HBO 100 W mercury lamp, and an FITC/Texas Red dual band filter set (Chroma Technology Corp., Brattleboro, VT, USA).

Nucleic acid preparation

Genomic DNA was prepared from *M. intracellulare* (ATCC no. 13950) and *M. bovis* (ATCC no. 35734). Cells grown in Dubos broth were washed in PBS, sedimented by centrifugation and stored at −80°C. Cell lysates were prepared by resuspending cell pellets (10 mg) in 300 μL of 370 mM NaCl and vortexing vigorously for 2 minutes in the presence of approximately 50 μL of acid-washed sand and 300 μL of buffer-saturated phenol, pH 8.0 (Gibco/BRL, Paisley, UK). After phase separation (10 min at 15000 × g) the aqueous phase was reextracted once with buffer-saturated phenol (pH 8.0), once with buffer-saturated phenol:chloroform:isoamylalcohol (25:24:1, pH 8.0)

(Gibco/BRL) and once with chloroform. Nucleic acids were precipitated with ethanol and recovered by centrifugation (10 min at 15000 × g at 4°C). After washing with 70% ethanol each nucleic acid pellet was redissolved in 50 μL TE-buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA).

PCR amplification of rDNA for sequencing

The design of oligonucleotide primers for PCR amplification of *M. intracellulare* and *M. bovis* 23S rDNA sequences was based on published sequences of *M. avium* (GenBank accession no. X74494) and *M. tuberculosis* (GenBank accession no. Z73902) 23S rDNA. For direct sequencing of PCR products, one primer in each pair was tagged with biotin at the 5′ end. PCR products for cloning were generated with primers lacking 5′-modifications. The PCR reaction mixtures included 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 500 μM of each oligonucleotide primer, 200 mM each of dNTPs, and 2.5u Taq polymerase (Promega, Madison, WI, USA) in 50 μL reaction volumes. Cycling conditions were as follows: 35 cycles at 94°C (1 min), 60°C (1 min), 72°C (1 min), followed by a final 10 minute elongation step at 72°C. The thermal cycler used was Omn-E (Hybaid, Middlesex, UK). All PCR amplification reactions gave rise to strong singular bands when analysed by agarose gel electrophoresis.

Cloning of PCR products for sequencing

PCR products were cloned in the pCR 2.1 vector using the TA Cloning Kit from Invitrogen (Carlsbad, CA, USA): one microlitre of each PCR product was mixed with 50 ng of the pCR 2.1 vector and incubated for 16 hrs at 14°C in the presence of 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 0.1 mg/mL bovine serum albumin, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine and 1 μL (4 u) T4 DNA ligase (Invitrogen). Following ligation, 2 μL of each reaction were mixed with 50 μL of competent TOP10 F′ cells (Invitrogen), and incubated for 30 min on ice. The cells were heat-shocked at 42°C for 30 seconds and placed on ice. The cells were transferred to 250 μL SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 1 hour at 37°C with shaking. Fifty microlitres from each transformation were spread on LB agar plates (1% Tryptone, 0.5% yeast extract, 170 mM NaCl, 1.5% agar) containing 100 μg/mL carbenicillin and incubated for 16 hours at 37°C. Colonies were picked and grown in 5 mL LB medium supplemented with 100 μg/mL carbenicillin for 16 hours at 37°C with shaking. Plasmids were prepared from the cultures using QIAprep spin-columns (Qiagen, Hilden, Germany).

Preparation of single-stranded PCR products for sequencing

Streptavidin-coated Dynabeads (Dynal, Norway) (200 μg/DNA preparation) were washed in 1 × binding and washing buffer (B&W buffer) containing 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA and 1 M NaCl, and resuspended in 40 μL 2 × B&W buffer. An equal volume of crude biotinylated PCR product was added and incubated for 15 minutes at room temperature. The beads were immobilised at the side of the tube by use of a magnet, and the supernatants were removed. After washing in 1 × B&W buffer, beads were resuspended in 0.1 M NaOH in order to denature the captured DNA. The beads were subsequently washed once in 0.1 M NaOH and once in TE buffer, and finally resuspended in 20 μL H_2O. Twenty microlitres of bead-bound single-stranded PCR product were used for each sequencing reaction.

DNA sequencing

In all cases, sequences were determined for both strands using cy5-labelled oligonucleotide primers (DNA technology, Århus, Denmark) and a 7-deaza-dGTP Thermo Sequenase cycle sequencing kit (Amersham, Little Chalfont, UK). Sequences were read using an ALFexpress automated sequencer and ALFwin (v. 1.10) software (Pharmacia Biotech, Uppsala, Sweden). In the case of plasmid-cloned material, sequences were determined from 10 individual clones from each PCR product. Sequencing of single-stranded PCR products was performed using products from four identical PCR reactions.

DNA sequence alignment and probe sequence selection

Sequence processing was done using computer software from DNASTAR (Madison, WI, USA). Alignments of mycobacteria 16S and 23S rDNA sequences, respectively, were done using the Megalign (v. 3.12) alignment tool. Up to one hundred sequences were aligned at a time (data not shown). For the alignment of mycobacteria 23S rDNA sequences, the sequences from *M. intracellulare* and *M. bovis* were obtained as described above.

PNA probes that are complementary to distinctive mycobacteria rRNA sequences were designed with due regard to secondary structures. Probe sequences were optimized in this respect using the PrimerSelect (v. 3.04) programme (DNASTAR). As a control of sequence specificity, all probe sequences were subsequently matched with the GenBank and EMBL databases using BLAST[14] sequence similarity searching at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

RESULTS

Probes used for direct detection of mycobacteria were synthesized as fluorescein-labelled PNA 15-mers of

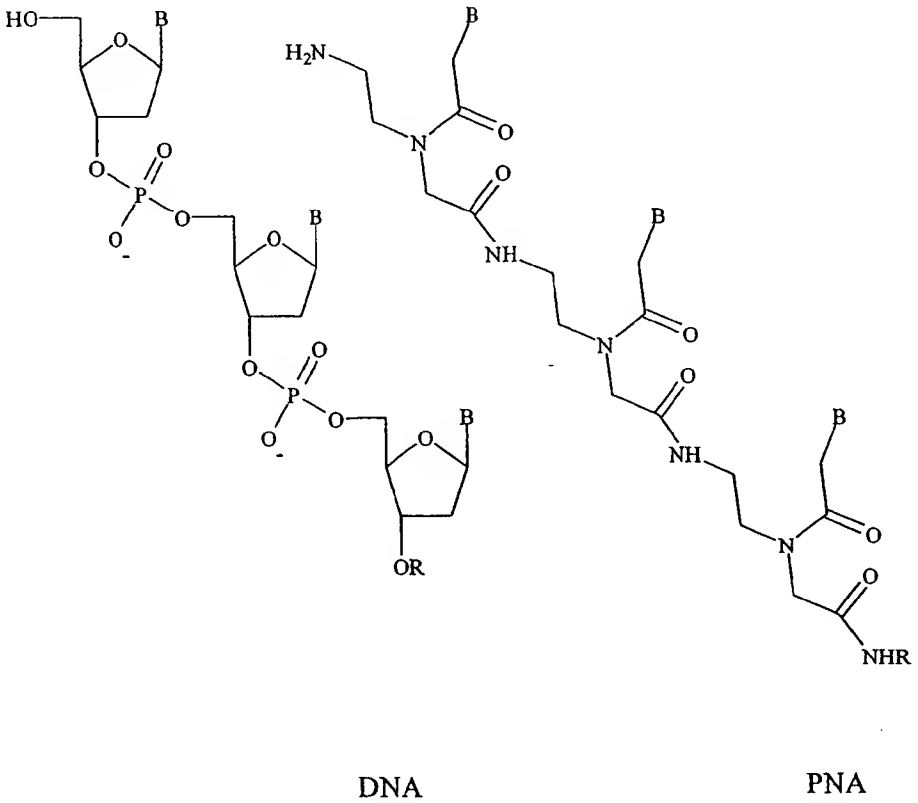

Figure 1 Chemical structure of DNA and PNA: as indicated, the PNAs consist of a polyamide backbone of *N*-(2-aminoethyl) glycine units to which nucleobases are covalently attached. B indicates a nucleobase (adenine, cytosine, guanine or thymine).

the structure shown in Figure 1. As indicated, the PNA counterpart of the sugar phosphate backbone of DNA and RNA is a polyamide formed by repetitive units of *N*-(2-aminoethyl)-glycine. Nucleobases are attached to this backbone to provide a molecular design that allows PNA to hybridize in a sequence-specific manner to complementary DNA or RNA sequences. By aligning the sequences of 23S and 16S rRNA from a number of mycobacterium species, rRNA sequences that are distinctive of *M. tuberculosis* and *M. bovis*, both members of the *M. tuberculosis* complex (MTC), were identified. Figure 2a shows an alignment of partial mycobacteria 23S rRNA sequences from the species indicated. The sequence of *M. tuberculosis* rRNA included in the Figure corresponds to position 3389-3419 of GenBank accession no. Z73902. Above the alignment is shown the antiparallel hybridization of the fluorescein-labelled PNA probe, OK682, used for detection of mycobacteria of the *M. tuberculosis* complex. Figure 2b shows an alignment of partial mycobacterium 16S rRNA sequences. Here, the sequence of *M. avium* corresponds to position 1101 to 1131 of GenBank accesssion no. M61673. The antiparallel hybridization of the fluorescein-labelled PNA probe, OK623, used for detection of non-tuberculous mycobacterium species, is shown above the alignment.

A BLAST sequence similarity search showed that the probe sequence of OK682 is not complementary to any published bacterial rRNA sequence, except those of *M. tuberculosis* and *M. bovis*, whereas the probe sequence of OK623 is complementary to published rRNA sequence of *M. avium*, *M. intracellulare*, *M. paratuberculosis*, *M. abscessus*, *M. kansasii*, *M. chelonae*, *M. malmoense*, *M. gastri*, *M. gordonae*, *M. szulgai*, *M. simiae*, *M. scrofulaceum*, as well as rRNA sequences of Actinomyces species and Rickettsia species.

Initially, the reactivity and specificity of the two probes were tested by the fluorescence in situ hybridization method using smears of *M. tuberculosis*, *M. bovis*, *M. avium*, *M. intracellulare*, and *M. kansasii*. From the results shown in Figure 3, it is apparent that *M. tuberculosis* and *M. bovis*, both members of the *M. tuberculosis* complex, were detectable after incubation with the fluorescein-labelled PNA probe, OK682. By contrast, neither *M. avium*, *M. intracellulare*, nor *M. kansasii* were detected after incubation with the same probe. Instead, these three non-tuberculous mycobacterium species could be detected using the fluorescein-labelled PNA probe, OK623. Neither *M. tuberculosis* nor *M. bovis* were detected with this probe. It is also apparent from Figure 3 that the detected cells of mycobacteria retain their morphology.

The results obtained using sputum from smear-

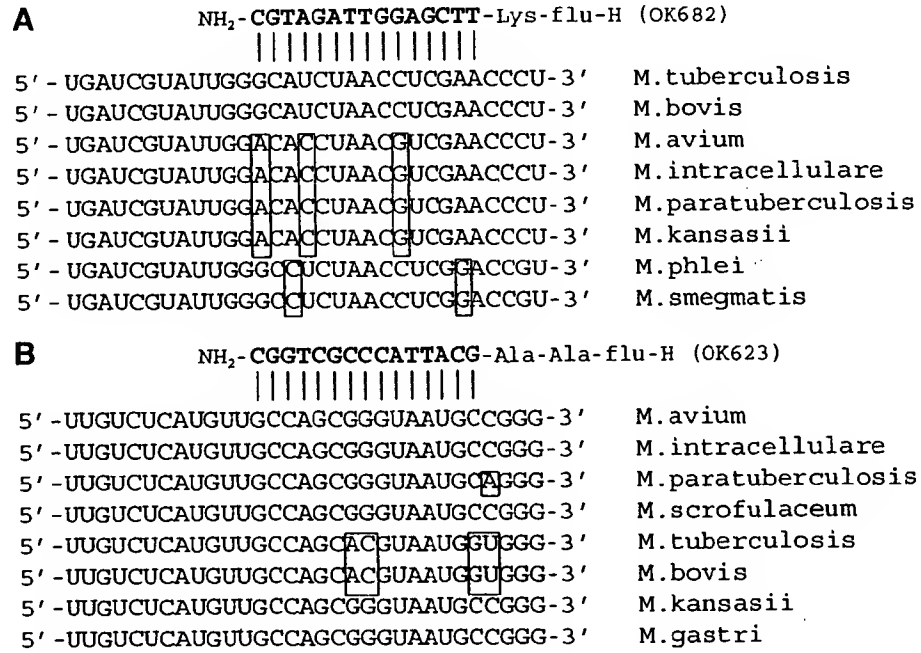

Figure 2 Alignments of mycobacterium rRNA sequences for probe detection: (A) Alignment of 23 S rRNA sequences of *M. tuberculosis* (Z73902), *M. bovis* (own sequence), *M. avium* (X94494), *M. intracellulare* (own sequence), *M. paratuberculosis* (X74495), *M. phlei* (X74493), *M. kansasii* (Z17212), and *M. smegmatis* (Y08453). OK682 targeting the 23 S rRNA of *M. tuberculosis* and *M. bovis* was labelled with 5(6)-carboxyfluorescein (flu) at the ε-aminogroup of lysine (lys). (B) Alignment of 16 S rRNA sequences of *M. tuberculosis* (X52917) *M. bovis* (M20940), *M. avium* (M61673), *M. intracellulare* (X52927), *M. paratuberculosis* (M61680), *M. scrofulaceum* (X52924), *M. kansasii* (X15916), and *M. gastri* (X52919). OK623 targeting the 16 S rRNA of *M. avium, M. intracellulare, M. paratuberculosis, M. scrofulaceum, M. kansasii*, and *M. gastri* is labelled with 5(6)-carboxyfluorescein (flu) at the β-aminogroup of alanine (ala). GenBank accession numbers of previously published sequences are shown in brackets.

positive cases of clinically suspected cases of *M. tuberculosis* infection are shown in Figure 4. *M. tuberculosis* is clearly detectable in these clinical samples with minimum background staining.

DISCUSSION

The discovery of PNA has raised a number of novel possibilities relating to molecular diagnostics. Some examples are illustrated in a range of reported molecular biology research applications.[15–23] Recent examples include application of PNA probes for FISH-based detection of telomere sequences in metaphase and interphase chromosomes,[24] and for in situ-based detection of immunoglobulin kappa light chain mRNA in paraffin sections.[25]

We have shown the potential of short, labelled PNA oligomers as a powerful means of identifying different species of mycobacteria directly in smears of cultured bacteria and sputum samples by FISH. The method presented here provides a combination of the high specificity offered by molecular techniques and the simplicity of direct microscopy.

The conditions for hybridization and post-hybridization wash are similar to those previously optimized for in situ hybridization using PNA probes,[25] with the exception of Triton X-100 included in both the hybridization buffer and wash solution. The main difference between this PNA in situ hybridization protocol and traditional DNA in situ hybridization protocols is 1) low salt concentration—10 mM NaCl (PNA hybridization is known to be salt-independent);[26] 2) shorter probes—15-mer: the Tm of a 15-mer PNA probe, according to the literature, is expected to be in the range of 60–70°C;[27] 3) high pH in the wash buffer (this is to avoid unspecific binding of the non-charged probe by deprotonation of the T-residues and fluorescein); and 4) a non-labelled, non-target PNA probe is added to the hybridization solution instead of non-labelled, non-target DNA or RNA, i.e., yeast tRNA, in order to prevent unspecific binding of the specific probe. In addition, the high chemical stability of PNA probes suggests that PNA probes are stable in the hybridization buffer at working concentration. Indeed, such ready-to-use PNA solutions for in situ hybridization are now commercially available (EBER PNA, DAKO).

Target amplification procedures for identification of *M. tuberculosis* in smear-positive sputum samples have recently been commercialized and approved by

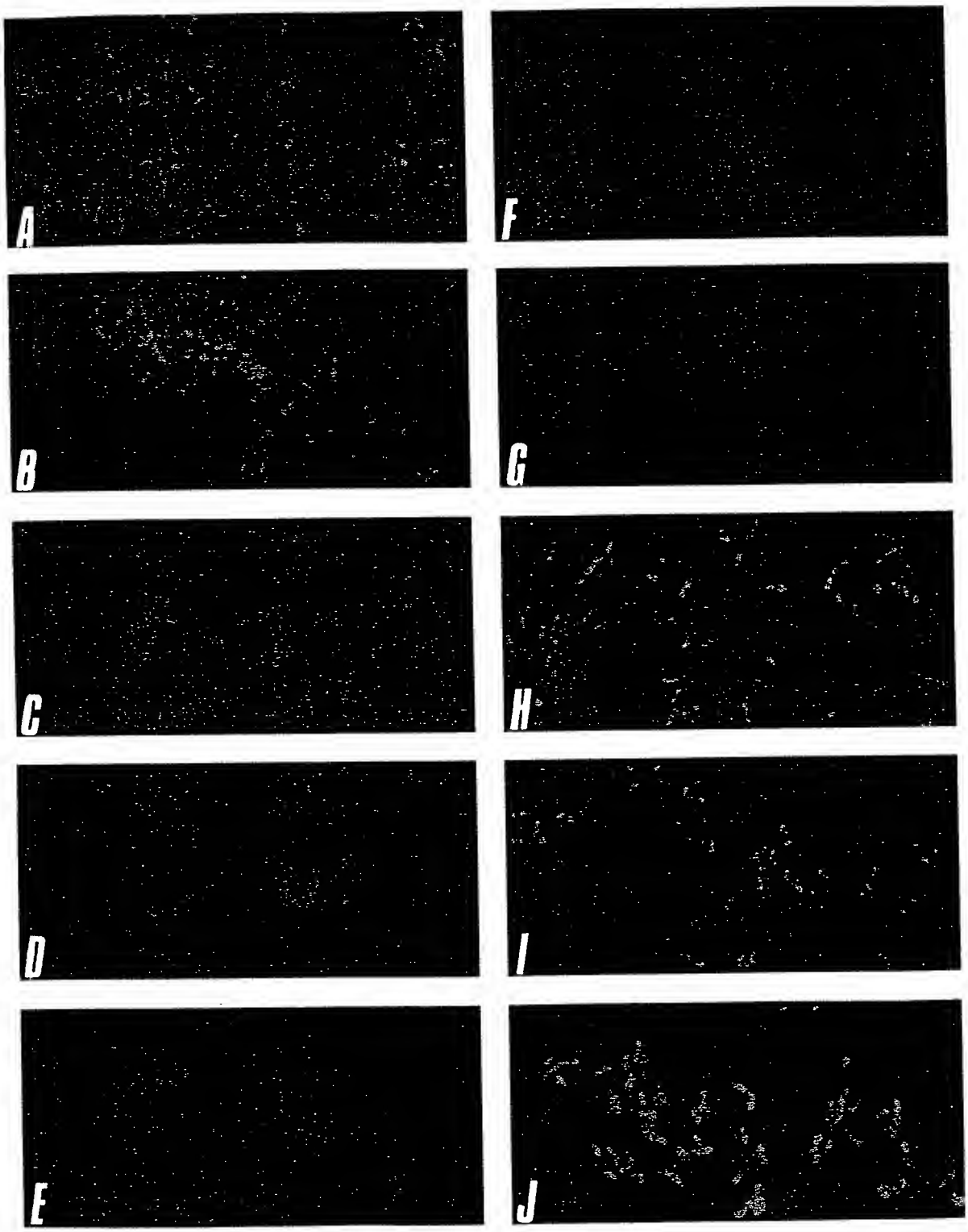

Figure 3 Mycobacterium species: (A) *M. tuberculosis* (B) *M. bovis* (C) *M. avium* (D) *M. intracellulare* (E) *M. kansasii* analysed with the PNA probe, OK 682, in a concentration of 100 nM. (F) *M. tuberculosis* (G) *M. bovis* (H) *M. avium* (I) *M. intracellulare* (J) *M. kansasii* analysed with the PNA probe, OK 623, in a concentration of 25 nM (magnification: ×1333).

the US Food and Drug Administration.[28,29] These tests require the use of costly chemicals and specialized instrumentation, and are thus amenable for use in large, specialized TB laboratories rather than in small standard laboratories.[30] By contrast, a routine test using FISH for confirmation of smear-positive cases of tuberculosis may be applicable to a wider range of laboratories.

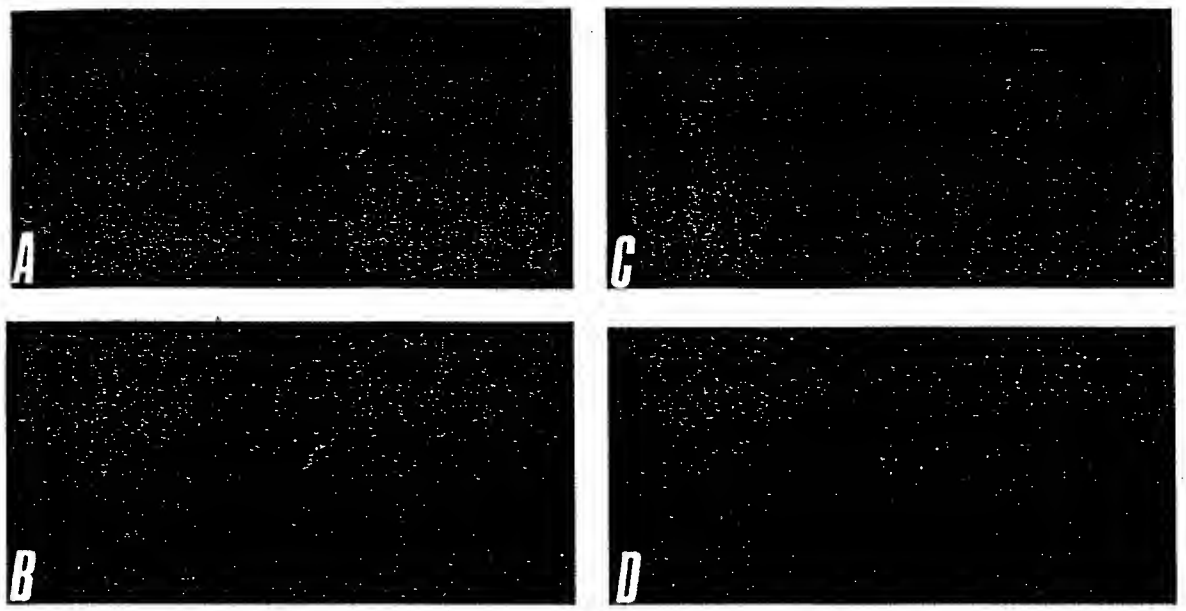

Figure 4 Sputum smears: (A–B) and (C–D). Two smear-positive sputum samples analysed with the PNA probe, OK 682, in a concentration of 100 nM (magnification: ×1333).

The fluorescence in situ hybridization method described in this study for direct detection of *M. tuberculosis* in clinical specimens was designed for routine use in clinical microbiology laboratories. It can be conducted while retaining the morphology of the target mycobacteria on both sputum smears and smears of cultured bacteria. This combination of signal and morphology is an important advantage of the present in situ hybridization method. Increased specificity as compared to current staining methods for AFB is achieved by targeting specific sequences of mycobacterial rRNA. Furthermore, the use of probes targeting rRNA makes direct detection possible without any amplification steps, since each mycobacterium cell contains hundreds to thousands of rRNA molecules.[32]

Direct identification of *M. tuberculosis* in smear-positive sputum samples by PNA-based FISH combines the morphological advantages of current staining methods with concomitant species identification. This will allow clinical microbiology laboratories to benefit from the advantages offered by molecular techniques to provide crucial information pertaining to treatment and patient management.

Studies are being initiated to establish diagnostic sensitivity and specificity data for this new diagnostic method, and other PNA probes, including those targeting mycobacteria in the *M. avium* complex as well as other species, are being evaluated.

Acknowledgements

The excellent technical assistance of Jani Hagemann, Ulla Søborg Larsen, and Somjai Mateeyonpiriya is greatly appreciated. Anne M Maarbjerg's aid in the synthesis of PNA oligomers is also acknowledged.

References

1 Raviglione M C, Snider D E, Kochi A. Global epidemiology of tuberculosis, morbidity, and mortality of a worldwide epidemic. JAMA 1995; 273: 220–226.
2 Reichman L B. Tuberculosis elimination—what's to stop us? Int J Tuberc Lung Dis 1997; 1: 3–11.
3 Centers for Disease Control and Prevention. Initial therapy for tuberculosis in the era of multidrug resistance—recommendation of the Advisory Council for the Elimination of Tuberculosis. MMWR, Morbidity and Mortality Weekly Report 1993; 42(RR-7): 1–8.
4 Kent P T, Kubica G P. Public health mycobacteriology. A guide for level III laboratories. Atlanta, GA: US Department of Health and Human Services, Centers of Disease Control, 1985.
5 Buchardt O, Egholm M, Nielsen P E, Berg R H. Peptide nucleic acids. PCT Patent Application No. WO 92/20702. 1992.
6 Buchardt O, Egholm M, Nielsen P E, Berg R H. The use of nucleic acid analogues in diagnostics and analytical procedures. PCT Patent Application No. WO 92/20703. 1992.
7 Nielsen P E, Egholm M, Berg R H, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 1991; 254: 1497–1500.
8 Egholm M, Buchardt O, Christensen L, et al. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 1993; 365: 566–568.
9 Stender H, Lund K, Andresen T. Novel probes for the detection of mycobacteria. PCT Patent Application No. PCT/DK97/00425. 1997.
10 Arnoldi J, Schlüter C, Duchrow M, et al. Species-specific assessment of *Mycobacterium leprae* in skin biopsies by in situ hybridization and polymerase chain reaction. Lab Invest 1992; 66: 618–623.
11 Thompson C T. Detection of mycobacteria. US Patent No. 5,582,985. 1994.
12 Murray C J L, Styblo K, Rouillon A. Tuberculosis in developing countries: burden, intervention and cost. Bull Int Union Tuberc Lung Dis 1990; 65(1): 6–24.
13 Sambrook J, Fritsch E F, Maniatis T. Molecular cloning, a lab-

oratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989.
14 Altschul S F, Gish W, Miller W, Myers E W, Lipman D J. Basic local alignment search tool. J Mol Biol 1990; 215: 403–410.
15 Boffa L C, Carpaneto E M, Allfrey V G. Isolation of active genes containing CAG repeats by DNA strand invasion by a peptide nucleic acid. Proc Natl Acad Sci USA 1995; 92: 1901–1905.
16 Demers D B, Curry E T, Egholm M, Sozer A C. Enhanced PCR amplification of VNTR locus D1S80 using peptide nucleic acid (PNA). Nucleic Acids Res 1995; 23: 3050–3055.
17 Demidov V V, Frank-Kamenetskii MD, Egholm M, Buchardt O, Nielsen P E. Sequence-selective double strand DNA cleavage by Peptide Nucleic Acid (PNA) targeting using nuclease S1. Nucleic Acids Res 1993; 21: 2103–2107.
18 Hanvey J C, Peffer N J, Bisi J E, et al. Antisense and antigene properties of peptide nucleic acids. Science 1992; 258: 1481–1485.
19 Møllegaard N E, Buchardt O, Egholm M, Nielsen P E. Peptide nucleic acid DNA strand displacement loops as artificial transcription promoters. Proc Natl Acad Sci USA 1994; 91: 3892–3895.
20 Nielsen P E, Egholm M, Berg R H, Buchardt O. Sequence-specific inhibition of DNA restriction enzyme cleavage by PNA. Nucleic Acids Res 1993; 21: 197–200.
21 Ørum H, Nielsen P E, Jorgensen M, Larsson C, Stanley C, Koch T. Sequence-specific purification of nucleic acid by PNA-controlled hybrid selection. BioTechniques 1995; 19: 472–479.
22 Veselkov A G, Demidov V V, Nielsen P E, Frank-Kamenetskii M D. A new class of genome rare cutters. Nucleic Acids Res 1996; 24: 2483–2487.
23 Vickers T A, Griffith M C, Ramasamy K, Risen L M, Freier S M. Inhibition of NF-κB-specific transcriptional activation by PNA strand invasion. Nucleic Acids Res 1995; 23: 3003–3008.
24 Lansdorp P M, Verwoerd N P, van de Rijke F M, et al. Heterogeneity in telomere length of human chromosomes. Hum Mol Genetics 1996; 5: 685–691.
25 Thisted M, Just T, Pluzek K-J, Petersen K H, Hyldig-Nielsen J-J, Godtfredsen S E. Detection of immunoglobulin kappa light chain mRNA in paraffin sections by in situ hybridization using peptide nucleic acid probes. Cell Vision 1996; 3: 358–363.
26 Thomac S, Sarkar M, Ratilainen T, et al. Ionic effects on the stability and conformation of peptide nucleic acid complexes. J Am Chem Soc 1996; 118: 5544–5552.
27 Jensen K K, Ørum H, Nielsen P E, Nordén B. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studies with the BIAcore technique. Biochemistry 1997; 36: 5072–5077.
28 Jones V, Alden M J, Curry J I, et al. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplification of rRNA. J Clin Microbiol 1993; 31: 2410–2416.
29 Moore D F, Curry J I. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by amplicor PCR. J Clin Microbiol 1995; 33: 2686–2691.
30 Doern G. Diagnostic mycobacteriology. Where are we today? J Clin Microbiol 1996; 34: 1873–1876.
31 Delong E F, Wickman G S, Pace N R. Phylogenetic strains: ribosomal RNA-based probes for the identification of single cells. Science 1989; 243: 1360–1363.
32 Winder F G. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of mycobacteria. In: C Ratledge, J Stanford, eds. The Biology of the Mycobacteria, vol. 1. New York: Academic Press Inc, 1982: pp 353–438.

RÉSUMÉ

CADRE : Les sondes de peptides d'acide nucléique (PNA) conçues pour la détection spécifique des mycobactéries du complexe *Mycobacterium tuberculosis* (MTC) et d'autres espèces de mycobactéries non tuberculeuses (NTM), respectivement, sont aptes à pénétrer la paroi cellulaire mycobactérienne et ultérieurement de s'hybrider in situ avec la rRNA complémentaire.
OBJECTIF : Démontrer l'utilisation de sondes PNA marquées à la fluorescéine pour la détection et l'identification de *M. tuberculosis* dans les échantillons d'expectorations à bacilloscopie positive.
SCHÉMA : La sensibilité et la spécificité des sondes PNA ont été investiguées par hybridation in situ en fluorescence (FISH) en utilisant des cultures de souches mycobactériennes représentant les espèces des groupes MTC et NTM, respectivement.
RÉSULTATS : *M. tuberculosis* a été détecté par FISH utilisant des sondes PNA spécifiques marquées à la fluorescéine, directement dans les échantillons d'expectorations à bacilloscopie positive, sans modifier la morphologie cellulaire.
CONCLUSION : Les sondes PNA sont utilisables pour le diagnostic rapide de la tuberculose dans les cas à bacilloscopie positive.

RESUMEN

MARCO DE REFERENCIA : Las sondas de ácido nucleico peptídico (PNA) diseñadas para la detección específica del complejo *Mycobacterium tuberculosis* (MTC) y otras especies de micobacterias no tuberculosas (NTM), respectivamente, han demostrado ser capaces de penetrar en la pared de la célula micobacteriana y posteriormente hibridarse in situ al rRNA complementario.
OBJETIVO : Demostrar el uso de las sondas de PNA marcadas con fluoresceína para la detección y la identificación del *M. tuberculosis* en muestras positivas de esputo.
MÉTODO : Se investigaron la sensibilidad y la especificidad de las sondas de PNA por la hibridización in situ en fluorescencia (FISH) empleando cultivos de micobacterias del tipo MTC y NTM, respectivamente.
RESULTADOS : Se detectaron *M. tuberculosis* por FISH, utilizando sondas de PNA marcadas con fluoresceína, directamente en muestras de esputos positivos, sin cambiar la morfología de la célula.
CONCLUSIÓN : Las sondas de PNA permiten un diagnóstico rápido de tuberculosis en los casos con esputos positivos.